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Accumulation of dopamine by blood platelets from normal subjects and parkinsonian patients under treatment with L-DOPA

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Summary

1. Human blood platelets have been shown to take up dopamine by an energy-dependent, saturable process that is inhibited by 5-hydroxytryptamine (5-HT), desipramine and other drugs.

2. Platelets from parkinsonian subjects receiving oral L-DOPA also took up dopamine.

3. When the responses of normal and parkinsonian platelets were compared, the parkinsonian cells showed the following differences: increased affinity for the dopamine transport process; decreased equilibrium concentrations of dopamine after incubation for 90 min, and greater efflux of dopamine from loaded platelets during a 10 min incubation.

4. There were no differences in the uptake of 5-HT by parkinsonian platelets, but endogenous 5-HT was significantly reduced; ATP was normal.

5. In two out of three samples of platelets from parkinsonian subjects, traces of a dopamine-like substance were detected, but this finding requires confirmation.

6. If the platelet is a valid model for dopaminergic brain neurones, then the results described would suggest that dopamine uptake and storage may be abnormal in brain neurones in Parkinson's disease.

Introduction

Although it has been known for many years that platelets accumulate 5-hydroxytryptamine (5-HT) (Humphrey & Toh, 1954; Hardisty & Stacey, 1955) and noradrenaline (Born & Hornykiewicz, 1957; Born, Hornykiewicz & Stafford, 1958), the accumulation of dopamine has not been investigated. Recently Da Prada & Pletscher (1969) have demonstrated the uptake of dopamine by the subcellular 5-HT storage organelles isolated from rabbit platelets.

We have studied the uptake of dopamine by normal human platelets and by platelets from subjects suffering from Parkinson's disease and under treatment with L-DOPA. The changes in equilibrium concentrations and efflux of dopamine in parkinsonian platelets, compared with controls, as described in this paper may be of particular interest in view of similarities between platelets and brain neurones regarding ability to accumulate and store catecholamines and 5-hydroxytryptamine

(Paasonen, 1968 ; Pletscher, 1968 ; Page, 1968). This analogy may now be extended to include dopaminergic neurones, whose function is known to be impaired in Parkinson's disease. If the platelet model is valid, it may provide insight into dopamine uptake and storage processes in the brain in Parkinson's disease.

Methods

Subjects

Two groups were used. In the first experiments, in which the dopamine uptake process was characterized, normal volunteers aged 14–36 years ($23 \pm 1{\cdot}2$=mean $\pm$ standard error of the mean) were used. These will be referred to as " controls " in this paper. They were significantly younger ($P<0{\cdot}001$) than the other subjects.

In the subsequent experiments involving a comparison of the responses of platelets in Parkinson's disease with controls, thirty-one parkinsonian patients and twenty normal subjects were used. The ages of the parkinsonian patients ranged from 36 to 77 years, with a mean of $62{\cdot}6 \pm 1{\cdot}6$ years. All received daily doses of L-DOPA. The mean dose was $4{\cdot}0 \pm 1{\cdot}1$ g/day (range of 2·25 to 6·0 g). Their controls were either spouses or other close relatives living in the same household. They will be called " parkinsonian controls ". Their mean age was $53{\cdot}4 \pm 3{\cdot}8$ years, which was just significantly lower ($P<0{\cdot}05$) than the mean age of the parkinsonian group. No sex differences in platelet function were noted.

Preparation of platelets

Blood collection and platelet isolation was made by the method of Boullin & O'Brien (1969) using polycarbonate pipettes and other laboratory utensils. The number of platelets per ml of plasma was determined with a Model B Coulter Counter (Coulter Electronics, Inc. Hialeah, Florida).

Measurement of dopamine and 5-HT uptake

Dopamine uptake was measured by determination of the content of radioactivity of platelets incubated with (^{14}C-2-ethylamine)-dopamine (specific activity 55 mCi/mmol ; Radiochemical Centre, Amersham). In all experiments involving the accumulation of 5-HT by platelets, ^{14}C-5-HT creatinine sulphate (specific activity 56 mCi/mmol ; Radiochemical Centre, Amersham) was used. 1 ml samples of platelet-rich plasma were preincubated for 10 min at 37° C in 5% carbon dioxide in oxygen before addition of dopamine or 5-HT. After addition of drug, incubation was continued for 5 to 120 min. Incubation was stopped by cooling the incubation tubes to 2° C. The platelets were then separated from the plasma by centrifugation at 20,000 *g* for 5 min at 2° C, or at 8,000 *g* for 5 min if the cells were to be resuspended. Both centrifugation procedures removed more than 99·5% of the platelets from the plasma. The resulting platelet-poor plasma was decanted and saved for radiochemical or spectrophotofluorimetric assay. Traces of plasma remaining in the incubation tube were removed with a cotton-tipped applicator covered with tissue. The platelet pellet was then lysed in 1·0 ml of distilled water with a Biosonik 11 sonifier, at a setting of 50 (Bronwill Scientific, Rochester, New York).

The platelet 5-HT content was determined by spectrophotofluorimetry (Bogdanski, Pletscher, Brodie & Udenfriend, 1956) and by liquid scintillation spectrometry, using Triton-X 100 and toluene (1:2) and a Beckman LS-250 liquid scintillation

spectrometer. In some experiments ^{14}C-dopamine was extracted from platelets after sonification by use of cation exchange resin and aluminium oxide adsorption (Taylor & Laverty, 1969).

Efflux experiments

To measure the efflux of dopamine, the cells were first incubated with 10^{-6} or 10^{-5}M ^{14}C-dopamine for 90 min, separated from plasma by centrifugation at 8,000 *g* for 5 min, and the platelet-poor plasma decanted. After addition of sufficient plasma to bring the plasma volume to the original value, the cells were resuspended at 2° C by agitation on a vortex mixer. To allow for the quantity of dopamine trapped in the interstices between cells we incubated samples of platelet-rich plasma with ^{14}C-dopamine at 0° C or ^{14}C-carboxylic acid-inulin (specific activity 3·08 μCi/mg, New England Nuclear Corp., Boston, Mass.). The amount of radioactivity recovered in the platelet pellet was taken as the " trapped cell volume ". This value was 1·5 μl/10^{11} cells by both methods.

ATP measurement

For ATP determinations platelets were isolated and lysed by sonification as described above. ATP was determined in the platelet lysate by the method of Holmsen, Holmsen & Bernhardsen (1966).

Drugs

Dopamine ; N-ethyl maleimide ; ouabain (Calbiochem, Los Angeles, California) ; 6-hydroxydopamine hydrobromide ; 5-hydroxytryptamine creatinine sulphate (Regis Chemical Co., Chicago, Illinois) ; desipramine hydrochloride (Geigy Pharmaceuticals, Ardsley, New York) and noradrenaline bitartrate (Winthrop Labs., New York) were used.

Results

Platelet count

No significant differences were noted in the platelet count between the controls, parkinsonian and parkinsonian control groups (Table 1). Consequently, the differences to be described below cannot be attributed to variations in the number of platelets.

Uptake of dopamine by normal platelets

When platelets were incubated in plasma, a considerable accumulation of dopamine occurred and equilibrium platelet concentrations were attained after 90–120

TABLE 1. *Platelet counts in experimental subjects*

	Controls	Parkinsonian	Parkinsonian controls
	(platelet count $\times 10^8$ cells)		
M	3·80	3·51	3·17
S.E.	±0·31	±0·16	±0·25
n	14	31	12

Values are the mean ± standard error of the mean (M±S.E.) obtained in the number of subjects indicated (*n*).

min incubation (Fig. 1). The concentration of dopamine in platelets (C_i) at equilibrium was related to the concentration in plasma (C_o). There was a linear relationship between C_i/C_o and log C_o over a range of plasma concentrations from 10^{-7} to 10^{-5}M (Fig. 2), suggesting a saturable mechanism.

The high C_i/C_o ratios of up to 130/1 suggested that uptake was by an energy-dependent process against a concentration gradient. This was shown to be true when low temperature and metabolic inhibitors were found to produce considerable inhibition of dopamine uptake (Table 2). The possibility that dopamine utilized the 5-HT transport system to enter platelets was investigated by studying the effects

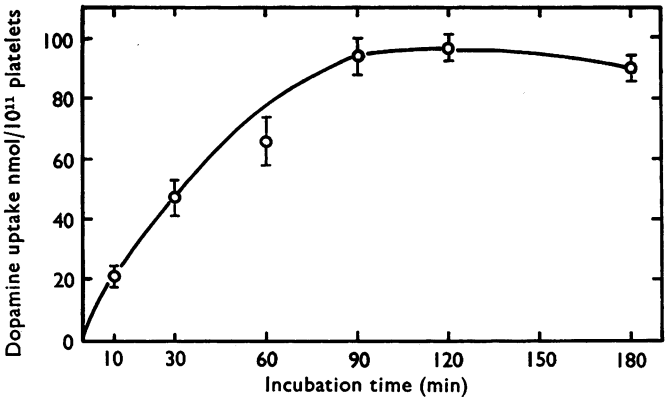

FIG. 1. Uptake of dopamine by normal human blood platelets. Platelet-rich plasma from nine control subjects was incubated with 10^{-6}M ^{14}C-dopamine for 10 to 180 min. Results are given as nmol dopamine/10^{11} platelets (mean value ± S.E. of the mean).

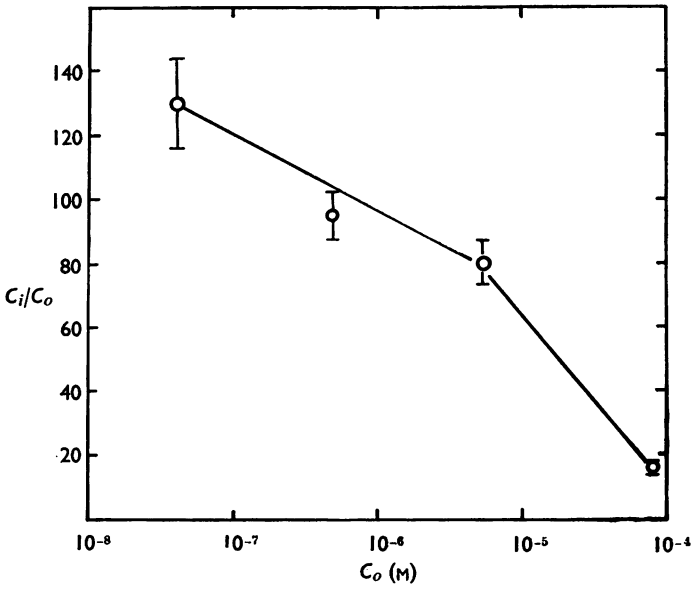

FIG. 2. Relationship of the ratio of platelet dopamine concentration nmol/ml packed platelets (C_i) : final plasma dopamine concentration at equilibrium nmol/ml (C_o) and log C_o. Values are mean ± S.E. of mean obtained in experiments with platelets from nine control subjects incubated with 10^{-7} to 10^{-4}M ^{14}C-dopamine for 90 min.

of 5-HT and a 5-HT uptake inhibitor, desipramine, on dopamine uptake. Table 2 shows that 5-HT and desipramine were potent inhibitors of uptake. Additionally, we found that noradrenaline was only moderately effective and that 6-hydroxydopamine was without effect. Thus 5-HT and dopamine were mutually antagonistic with regard to accumulation.

If, indeed, 5-HT and dopamine are transported into platelets by the same energy-requiring mechanism, both amines should compete for the transport system and act as inhibitors. Therefore, experiments were made to study the interactions of the two amines. Platelets were incubated with non-radioactive dopamine or 5-HT for 5 min, and then the other amine in the ^{14}C-labelled form was added. Uptake was then continued for 90 min. The K_i for inhibition of uptake was determined using concentrations of inhibitor ranging from 10^{-7} to 10^{-4}M. In three experiments both substances were found to be effective inhibitors, but 5-HT was a more potent inhibitor of dopamine uptake ($K_i = 5{\cdot}8 \times 10^{-6}$M) than dopamine was of 5-HT uptake ($K_i = 9{\cdot}6 \times 10^{-5}$M). This latter value is in close agreement with that found by Stacey (1961) when he studied the effect of dopamine on 5-HT uptake; 15×10^{-5}M dopamine caused 50% inhibition.

Uptake of dopamine by parkinsonian platelets

The extent of the experiments was limited by the number of parkinsonian patients available and the volume of blood that could be drawn, so only a few basic observations have been made. Parkinsonian platelets accumulated dopamine similarly to controls and the kinetics of uptake in experiments with a group of six subjects and six controls is given in Table 3. Since K_m for parkinsonian platelets is less than for controls, but V_{max} is the same for both groups (see also **Discussion**), $V_{control}$ should be less than $V_{parkinsonian}$. This is not evident from Fig. 3 and may possibly be due to an insufficient number of observations or insufficiently accurate determinations of V_{max} using results based on 10 min incubation rather than a shorter time. From the K_m values it appeared that parkinsonian platelets had a somewhat greater affinity for dopamine than the controls.

The time course of uptake of dopamine and 5-HT was compared in other experiments with seven parkinsonian subjects and five controls. The results are given in Fig. 3. The equilibrium concentration of dopamine in platelets of the parkinsonian group was reduced whereas 5-HT uptake at equilibrium was not changed.

TABLE 2. *Factors affecting the uptake of dopamine by platelets*

Inhibitor	Concentration (M)	Time (min)	Inhibition of dopamine uptake (%)
Low temp. (24° C)	—	—	75·6±0·2
N-ethylmaleimide	10^{-4}	30	98·5±3·2
Ouabain	10^{-4}	30	29·5±2·1
5-HT	10^{-5}	5	58·3±1·2
5-HT	10^{-4}	5	76·6±0·8
Desipramine	10^{-4}	30	94·8±3·2
Noradrenaline	10^{-4}	5	38·9±4·2
6-Hydroxydopamine	10^{-4}	30	16·9±2·2

The results shown are the mean±S.E. obtained in four to six experiments.
Inhibitory drugs were added to platelet-rich plasma 5 or 30 min before incubation with 10^{-6}M ^{14}C-dopamine for 90 min. The dopamine uptake in the absence of inhibitors was 692±37 nmol/10^{11} cells.

Further experiments were therefore made to determine equilibrium dopamine concentrations in twelve control and twelve subjects with Parkinson's disease using plasma concentrations of dopamine ranging from 10^{-8} to 10^{-5}M. Table 4 shows that equilibrium concentrations were significantly reduced in the parkinsonian subjects.

Efflux of dopamine from parkinsonian and control platelets

Using sixteen subjects with Parkinson's disease and seven control subjects we studied the efflux of dopamine from platelets loaded with ^{14}C-dopamine by incubation with 10^{-6}M for 90 min and then resuspended in dopamine-free plasma for up to 120 min.

In contrast to 5-HT, dopamine is lost rapidly from both normal and parkinsonian platelets. In both groups approximately 8·5% of the amount accumulated was lost during resuspension. This is about double the amount of 5-HT lost in comparable experiments with 5-HT-loaded platelets (Boullin, Coleman & O'Brien, 1969).

TABLE 3. *Kinetics of dopamine uptake by normal and parkinsonian platelets*

	$K_m \times 10^6$	V_{max} (nmol/min/10^{11} platelets)
Parkinsonian control	0·72±0·08	1·92±0·48
Parkinsonian	0·38±0·09	2·41±0·33

The results (mean±S.E.) were obtained using platelets from six parkinsonian patients and six parkinsonian control subjects. Platelets were incubated with 10^{-8} to 10^{-4}M ^{14}C-dopamine for 5 min. K_m and V_{max} were calculated according to the Michaelis-Menten equation used to describe saturable enzyme/substrate interactions.

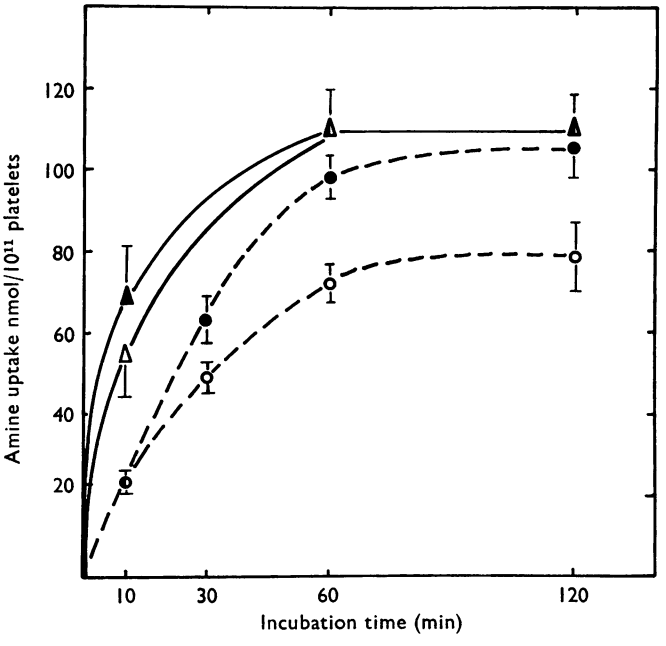

FIG. 3. Comparison of the time course of uptake of 5-HT and dopamine by platelets from parkinsonian and parkinsonian control subjects. Parkinsonian 5-HT, △; control 5-HT, ▲. Parkinsonian dopamine ○; control dopamine, ●. Platelets were incubated with 10^{-6}M ^{14}C-5-HT or ^{14}C-dopamine for 10 to 120 min. Values are the mean±S.E. of the mean of six parkinsonian and six parkinsonian control experiments.

Further losses of dopamine occurred when the platelets were reincubated. The efflux from parkinsonian platelets after 10 min incubation was significantly greater than controls (Fig. 4), though there was no significant difference in the efflux after 120 min incubation.

Attempts to find dopamine in parkinsonian platelets

In view of our findings that parkinsonian platelets take up dopamine *in vitro* it seemed likely that, *in vivo*, they might contain dopamine, derived from L-DOPA, but synthesized in other tissues and then transferred to the platelet via the circulation. In two of the first subjects studied, a dopamine-like substance was recovered from platelets isolated from 7 to 15 ml of platelet-rich plasma, following adsorption of the platelet extract on to alumina and elution with 0·2 N acetic acid (Taylor & Laverty, 1969). However, when the samples were analysed chromatographically (Laasberg & Shimosato, 1966), we were unable to obtain a spot that could be

TABLE 4. *Equilibrium concentrations of dopamine in parkinsonian and control platelets*

Plasma concentration (M)	10^{-8}	10^{-7}	10^{-6}	10^{-5}
	Platelet dopamine (nmol/10^{11} platelets)			
Control	1·35±0·08	12·7±0·8	106·8±5·5	323·8±27·1
Parkinsonian	0·98±0·08	10·5±0·4	73·6±5·8	308·6±27·6
P	<0·01	<0·05	<0·01	NS

The above results were obtained in experiments with platelets from twelve parkinsonian and twelve parkinsonian control subjects, incubated with ^{14}C-dopamine for 90 min. Values are the mean±S.E. NS, Mean parkinsonian value not significantly different from control value.

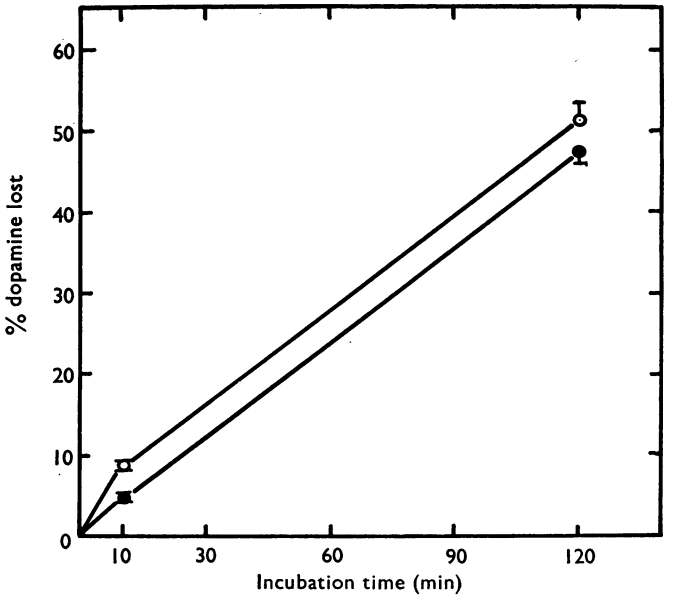

FIG. 4. Efflux of ^{14}C-dopamine from parkinsonian (○) and parkinsonian control (●) platelets. Cells were incubated with 10^{-6}M ^{14}C-dopamine for 90 min. They were then resuspended in drug-free plasma as described in **Methods** and reincubated for 10 or 20 min. The values given are the mean % loss of dopamine (±S.E. of the mean) for sixteen parkinsonian and seven parkinsonian control experiments. The resuspension procedure alone caused additional dopamine loss of 8·74±1·65% in the parkinsonian and 8·52±0·80% in the parkinsonian control experiments, which is not shown in the figure.

unequivocally distinguished from DOPA. Thus the fluorescent material in parkinsonian platelets could have been DOPA, dopamine or a mixture. Subsequently we made another attempt to isolate the dopamine-like substance, using platelets from 53 ml of plasma pooled from eight patients (approximately 2×10^{10} platelets), but we failed to detect any dopamine-like fluorescence. The figures given below for the "dopamine" content in the first two subjects studied are therefore purely tentative. Platelet content of a dopamine-like substance was 39·0 and 15·1 nmol/10^{11} platelets.

5-HT and ATP content of parkinsonian platelets

Platelet 5-HT and ATP were measured in fourteen parkinsonian subjects and nine parkinsonian controls. There was a significant reduction in 5-HT in the parkinsonian subjects, but no reduction in ATP (Table 5). The ratio ATP:5-HT was higher in the latter group, but not significantly so.

Discussion

Normal platelets

Normal platelets take up exogenous dopamine in a similar fashion and to a similar extent to 5-HT. 5-HT and dopamine are antagonistic with regard to accumulation and dopamine may use the same energy-dependent transport mechanism as that normally used by 5-HT, since uptake is blocked by low temperature, metabolic inhibitors, and other substances, like desipramine, which are known to inhibit the 5-HT transport process in platelets (Stacey, 1961).

In addition to being taken up, dopamine and 5-HT also act as inhibitors of uptake, 5-HT being a more potent inhibitor of dopamine than vice versa.

In their experiments on dopamine upake by subcellular storage organelles from rabbit platelets, Da Prada & Pletscher (1969) found that dopamine accumulation was 68% of 5-HT uptake. Our results with normal human platelets show that the two amines were accumulated equally (Fig. 3). In view of this similarity it was surprising that the retention patterns were not similar. Although Berneis, Pletscher & Da Prada (1969) and Berneis, Da Prada & Pletscher (1969) have shown that both dopamine and 5-HT form micelles with ATP, the present work and Da Prada & Pletscher's experiments referred to above suggest that dopamine has less affinity for ATP than has 5-HT.

Parkinsonian platelets

Dopamine appeared to have greater affinity for the transport mechanism in parkinsonian platelets than in controls, since the value for K_m in the parkinsonian group was half the normal value (Table 3). But as V_{max} was the same in both

TABLE 5. *5-HT and ATP in normal and parkinsonian platelets*

	Substance/10^{11} cells		
	5-HT (nmol)	ATP (μmol)	ATP:5-HT
Parkinsonian controls	492±80	2·78±0·68	6·74±1·63
Parkinsonian subjects	311±32*	2·21±0·27	8·56±1·61

The above values (mean±S.E.) were obtained in six parkinsonian and six parkinsonian control subjects. * The 5-HT content of parkinsonian platelets was significantly lower ($P<0·01$) than the control value; all other values were not significantly different.

groups, further work will be required on the mechanism of dopamine transport in parkinsonian platelets. In this regard, the possibility of L-DOPA treatment exerting a remedial action on defective amine transport systems is intriguing.

Two lines of evidence suggest abnormal dopamine binding in parkinsonian platelets. First, the significantly greater efflux from loaded cells observed during the first 10 min of incubation (Fig. 4); second, the decreased concentrations at the steady state (Table 4). These observations may be related because tissue concentrations represent a dynamic equilibrium between uptake and efflux; this may explain why no difference in efflux was seen after 120 min incubation. At that time efflux would be expected to be less as the C_i/C_o ratio was greatly reduced. At present we cannot draw any conclusion regarding the nature of the binding defect, but as there was no reduction in ATP, changes in complex formation seem unlikely.

Regarding the attempts to demonstrate dopamine in parkinsonian platelets, little can be said except that the possibility exists that the cells can take up dopamine *in vivo*. If the tentative values given are correct, then platelets in parkinsonian patients under treatment with L-DOPA contain dopamine to the extent of 1/10 to 1/20 of their 5-HT content.

There is no abnormality in the uptake of 5-HT by platelets from patients with Parkinson's disease (Fig. 3). The reduction in endogenous 5-HT could be due to either *in vivo* inhibition of 5-HT uptake resulting from increased plasma concentrations of dopamine, or competition between L-DOPA and L-5-hydroxytryptophan for the enzyme L-aromatic amino-acid decarboxylase. The latter would result in increased dopamine, but decreased 5-HT synthesis, probably in the brain and intestine.

In considering these results in relation to Parkinson's disease, it is important to remember that all subjects were treated with high doses of L-DOPA. Consequently any of the changes described in this paper may have been due to the effect of the drug alone or to metabolic effects resulting from treatment. Alternatively the responses may have been ameliorated by the drug and thus be worse in untreated parkinsonian patients. Unfortunately untreated parkinsonian patients are not available for us to study at present.

On the basis of the current views of the biochemical defects occurring in Parkinson's disease and the mechanism of action of L-DOPA (see Barbeau, 1969), the effects described here seem unlikely to have been entirely a result of a pharmacological effect of the drug. Thus the platelet may be a model for dopaminergic neurones in the brain, and, in addition to decreased dopamine synthesis, the biochemical lesions may include defective binding and excessive loss of dopamine.

Addendum

Recently we have obtained blood from three untreated parkinsonian subjects, and have examined several of the parameters described in the paper.

The endogenous 5-HT content was normal in the untreated patients (493 ± 52 nmol/10^{11} platelets). The dopamine accumulation was also normal in platelets incubated with 10^{-6}M dopamine for 120 min ($109{\cdot}6 \pm 6{\cdot}9$ nmol/10^{11} cells).

On the other hand the efflux of dopamine from loaded platelets was enhanced. The losses were $14{\cdot}7 \pm 1{\cdot}0\%$ after 10 min and $55{\cdot}9 \pm 0{\cdot}6\%$ after 120 min incubation. In comparison with the results in Fig. 4, efflux was significantly greater in untreated

parkinsonian patients compared with controls; in comparison with the treated patients, the efflux after 10 min incubation was also significantly greater ($P<0.01$), but after 120 min incubation there was no difference.

These very limited observations argue in favour of the view that the reduction in endogenous 5-HT and decreased dopamine uptake in treated parkinsonian patients is a result of DOPA administration rather than a consequence of the disease state itself. On the other hand, the increased efflux of dopamine after 10 min in the three untreated subjects suggests that in Parkinson's disease there may well be an increased efflux, and therefore diminished binding of dopamine in the platelets and possibly in other cells.

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